

Effect of Biofield Energy Treatment on Bone Cell Proliferation and Differentiation for the Assessment of its Potential to Improve Bone Health

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Abstract

The bone health is an important part of healthy-life and longevity in general. The present study was investigated to see the effect of the Biofield Energy Healing (The Trivedi Effect®) on the human bone osteosarcoma cells - MG-63 (ATCC® CRL-1427™) for the assessment of bone cell proliferation and differentiation *in vitro*. The study parameters were assessed using cell viability by MTT, collagen synthesis, and alkaline phosphatase (ALP) on bone health using ELISA-based assay. The cell viability assay data showed significant response in all the tested groups; while in the Biofield Energy Treated group supplemented with 10% charcoal-dextran treated fetal bovine serum (CD-FBS) (G3) showed better response (increased 63%) in terms of cells proliferation compared to the untreated cell group (G1). The level of ALP was increased by 32% in the G3 group compared to the untreated cells group (G1). Additionally, the level of collagen synthesis was increased by 27% in the G3 group compared to the G1 group. The overall results demonstrated that the Biofield Energy Treatment has the potential for bone mineralization and bone growth as evident *via* increased levels of collagen and ALP. Therefore, the Biofield Energy Healing (The Trivedi Effect®) Treatment might be useful as a bone health promoter for various bone-related disorders like low bone density, osteogenesis imperfecta, and osteoporosis.

Keywords: Biofield Energy Healing; The Trivedi Effect®; Alkaline phosphatase; Collagen; Osteosarcoma cells - MG-63; β -Estradiol; MTT; ELISA

Abbreviations: MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS: Phosphate buffer saline; ELISA: Enzyme-linked immunosorbent assay; CD-FBS: Charcoal-dextran treated fetal bovine serum; ALP: Alkaline phosphatase; NCCAM: National Center for Complementary and Alternative Medicine.

Introduction

Bone formation needs differentiated and active osteoblasts for the synthesis the extracellular matrix, which enhanced the process of mineralization [1]. The human osteosarcoma cell line (MG-63) has been used to

study the production of the bone-specific protein, osteocalcin. In the absence of any stimuli, MG-63 cells secreted very low level of osteocalcin [2]. From the literature, it was indicated that parathyroid hormone (PTH) and prostaglandin E2 (PGE2) inhibits osteocalcin secretion by a mechanism involving cAMP production. In contrast, an increase in extracellular calcium, which stimulate osteocalcin release. The regulation of osteocalcin secretion is mediated by the PTH and PGE2 in normal human bone cells, which can be produced in the human osteoblast-like cell line MG-63. Both cell cultures showed time- and dose-dependent stimulation of osteocalcin secretion in response to 1,25-dihydroxyvitamin D3 (1,25(OH)2D3) active form of vitamin D₃. Thus the human osteosarcoma cell line MG-63 is useful as an alternative osteoblast-like cell model to study the regulation of osteocalcin secretion [3]. Alkaline phosphatase (ALP) has many different functions in the many organisms and tissues and plays a major role in mineralization of bone tissues. More than 80 years ago, the high level of ALP expression in bone was observed indicating it's important for hard tissue formation. Scientific research demonstrated that ALP had increased the local concentration of inorganic phosphate (Pi), a concept known as the 'booster hypothesis' [4]. Different types of approaches such as molecular, biochemical, histochemical, and ultrastructural have been studied for the establishment of a temporal sequence of gene expression associated with the development of the bone cell phenotype in primary osteoblast cultures. The peak levels of expressed genes reflect a developmental sequence of bone cell differentiation characterized by three principal periods: proliferation, extracellular matrix maturation and mineralization [5]. ALP can play a key role in the process of bone mineralization. It is expressed in both bone and calcifying cartilage, an early stage of bone development. Later on, other gene (*e.g.* osteocalcin) is upregulated and simultaneously ALP expression had declined. ALP mainly acts in the initial phases of the mineralization process. The mechanisms through which ALP expression regulated are complex; a web of interlaced signaling pathways, controlling osteoblastic differentiation, chondrogenesis and ALP expression are the BMP/RUNX2 (CBAf1, AML3)/ Osterix system, and the WNT signaling cascade, which also interact with each other [6,7]. In today's world, Energy Therapy like Biofield Energy Healing has been widely used and recommended as an alternative method that has an impact on various properties of living organisms in a cost-effective manner [8]. The Trivedi Effect® - Biofield Energy Healing has been known to improve the potential beneficial effects in a broad spectrum field around the Globe. It improved the overall productivity of crops in agriculture and livestock

[9-12], positive impact on cancer [13,14], and altered characteristics features of microbes in the field of microbiology [15-18]. It also alters the structural, physical, and thermal properties of several metals and ceramics [19-21], causes genetic alteration in microbes [22,23], and improves various nutraceutical compounds in the areas of nutraceuticals [24,25] and biotechnology [26-28]. Many treatment strategies are available for the proliferation and differentiation of bone cells using synthetic drugs and chemicals. However, due to its high cost, anticipated toxicity, render it undesirable for the patients. Based on the impact of The Trivedi Effect® in various field, authors intended to explore the study of the Biofield Energy Healing on bone health in *in vitro* cellular model *i.e.*, human bone osteosarcoma cells - MG-63. Therefore, authors investigated the effect of Biofield Energy Healing (The Trivedi Effect®) on the human bone osteosarcoma cells - MG-63 (ATCC® CRL-1427™) for the assessment of bone proliferation and differentiation *in vitro*.

Materials and Methods

Chemicals and Reagents

Antibiotics solution (penicillin-streptomycin) and DMEM (phenol-red free) were procured from HiMedia, India. DMEM was procured from GIBCO, USA. Direct Red 80, 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium) (MTT), and β -estradiol (positive control) were purchased from Sigma Chemical Co. St. Louis, MO, USA. All the other chemicals used in this experiment were analytical grade procured from India.

Cell Culture and Maintenance

Human bone osteosarcoma cell line -MG-63 (ATCC® CRL-1427™) was used as test system in the present study. MG-63 cell line was maintained under DMEM growth medium for routine culture supplemented with 10% FBS. Growth conditions were maintained at 37°C, 5% CO₂, and 95% humidity and subcultured by trypsinisation followed by splitting the cell suspension into fresh flasks and supplementing with fresh cell growth medium. Three days before the start of the experiment (*i.e.*, day -3), the growth medium of near-confluent cells was replaced with fresh phenol-free DMEM, supplemented with 10% charcoal dextran stripped FBS (CD-FBS) and 1% penicillin-streptomycin [29].

Biofield Energy Healing Approach

An aliquot of MG-63 cells in a T-25 cell culture flask received Biofield Energy Treatment (The Trivedi Effect®)

under laboratory conditions for ~3 minutes from a distance of ~25 cm, at day 0. The energy transmission was done without touching the cells. Following Biofield Energy Treatment, the above T-25 flask was incubated for 48 hours with defined conditions along with the untreated cells.

Experimental Design

The tested cells were divided into three groups. Group 1 served as baseline control (untreated cells with 200 μ L of phenol-free DMEM supplemented with 10% CD-FBS). Group 2 served as positive control (untreated cells + 180 μ L of phenol-free DMEM with 10% CD-FBS + β -estradiol at three different concentrations such as 1, 10, and 100 nM). Group 3 was received Biofield Energy Treated cells in phenol-free DMEM medium supplemented with 10% CD-FBS.

Assessment of Cell Proliferation

The Biofield Energy Treated MG-63 cells as well as the untreated MG-63 cells were trypsinized, counted, and plated in wells of flat bottom in 96-well plates at the density corresponding to 5×10^3 cells/well/180 μ L of growth medium. Following respective treatments, the cells in the 96-well plates were incubated for 48 hours in a CO₂ incubator at 37°C, 5% CO₂, and 95% humidity. After 48 hours of incubation, the plate was taken out and 20 μ L of 5 mg/mL of MTT solution was added to all the wells followed by additional incubation for 3 hours at 37°C. The supernatant was aspirated and 150 μ L of DMSO was added to each well to dissolve formazan crystals. The absorbance of each well was read at 540 nm using Synergy HT microplate reader [30]. The percentage cell growth corresponding to each treatment was calculated using formula (1):

$$\% \text{ Cell growth} = \left[\frac{[(X-T_z)/(R-T_z)] \times 100}{\dots\dots\dots} \right] \dots\dots\dots (1)$$

Where, X = Absorbance of cells corresponding to positive control and test groups after 48 hours

R = Absorbance of cells corresponding to baseline group after 48 hours

T_z = Absorbance of untreated cells at time 0 hour

Positive control was run in parallel to the sample. Concentrations were determined, and the experiment was done in triplicates.

Assessment of Alkaline Phosphatase (ALP) Activity

The Biofield Energy Treated MG-63 cells as well as untreated MG-63 cells were trypsinized, counted and

plated in wells of flat bottom 96-well plates at the density corresponding to 10×10^3 cells/well/180 μ L of growth medium. Following respective treatments, the cells in the above plate were incubated for 48 hours in a CO₂ incubator at 37°C, 5% CO₂, and 95% humidity. After 48 hours of incubation, the plate was taken out and processed for measurement of ALP enzyme activity. The cells were washed with 1X PBS and lysed by freeze-thaw method *i.e.*, incubation at -80°C for 20 minutes followed by incubation at 37°C for 10 minutes. To the lysed cells, 50 μ L of substrate solution *i.e.*, 5 mM of *p*-nitrophenyl phosphate (*p*NPP) in 1M diethanolamine and 0.24 mM magnesium chloride (MgCl₂) solution (pH 10.4) was added to all the wells followed by incubation for 1 hour at 37°C. The absorbance of the above solution was read at 405 nm using Synergy HT microplate reader (Biotek, USA). The absorbance values obtained were normalized with substrate blank (*p*NPP solution alone) absorbance values. The percentage increase in ALP enzyme activity with respect to the untreated cells (baseline group) was calculated using formula (2):

$$\% \text{ Increase} = [(X-R)/R] \times 100 \dots\dots\dots (2)$$

Where, X = Absorbance of cells corresponding to positive control and test groups

R = Absorbance of cells corresponding to baseline group (untreated cells)

Assessment of Collagen Synthesis

The Biofield Energy Treated MG-63 cells as well as untreated MG-63 cells were trypsinized, counted and plated in wells of 48-well plates at the density corresponding to 10×10^3 cells/well/0.5 mL of growth medium. Following respective treatments, the cells in the above plate were incubated for 48 hours in a CO₂ incubator at 37°C, 5% CO₂, and 95% humidity. After 48 hours of incubation, the plate was taken out and the amount of collagen accumulated in MG-63 cells corresponding to each treatment was measured by Direct Sirius red dye binding assay. In brief, the cell layers were washed with PBS and fixed in Bouin's solution (5% acetic acid, 9% formaldehyde and 0.9% picric acid) for 1 hour at room temperature (RT). After 1 hour of incubation, the above wells were washed with milliQ water and air dried. The cells were then stained with Sirius red dye solution for 1 hour at RT followed by washing in 0.01 N HCl to remove unbound dye. The collagen dye complex obtained in the above step was dissolved in 0.1 N NaOH and absorbance was read at 540 nm using Biotek Synergy HT microplate reader. The level of collagen was extrapolated using standard curve obtained from purified Calf Collagen

Bornstein and Traub Type I (Sigma Type III). The percentage increase in collagen level with respect to the untreated cells (baseline group) was calculated using formula (3):

$$\% \text{ Increase} = ((X-R)/R) \times 100 \dots \dots \dots (3)$$

Where, X = Collagen levels in cells corresponding to positive control and test groups

R = Collagen levels in cells corresponding to baseline group (untreated cells)

Statistical Analysis

Data analysis was performed with SigmaPlot Statistical Software (Version 11.0). Differences between means (in triplicates) were assessed for statistical differences using one-way analysis of variance (ANOVA) and *post-hoc* analysis was done by Dunnett's test. $p \leq 0.05$ was

statistically significant. The results are shown as mean \pm standard deviation (SD).

Results

Assessment of Cells Viability by MTT Assay

The effect of the Biofield Energy Healing on the proliferation of human bone osteosarcoma cell was examined after 48 hours using MTT assay are shown in Table 1. The cell viability was significantly ($p \leq 0.001$) increased by 63% in the Biofield Energy Treated cells supplemented with 10% CD-FBS (G3) compared to the untreated cells (G1). The cell viability in the positive control group (G2) was significantly increased by 19%, 68%, and 53% at the concentration of 1, 10, and 100 nM, respectively compared to the G1 group. The cell viability was significantly increased in all the tested groups compared to the untreated cells group (G1).

Group	G1		G2			
	Time (T0)	Time (72h)	1 nM	10 nM	100 nM	G3
Absorbance	0.527 \pm 0.054	0.910 \pm 0.034	0.984	1.170 \pm 0.101	1.114 \pm 0.033	1.150 \pm 0.048
			\pm			
			0.041			
% Cell Proliferation w.r.t. T0	0	100	119	168***	153***	163*

G1: Untreated cells; G2: Positive control (β -Estradiol) at the concentrations of 1, 10 and 100 nM. G3: Biofield Energy Treated cells. All the groups were included phenol-free DMEM supplemented with 10% CD-FBS. Values are represented as mean \pm SD and the experiment was carried out in triplicates. *** $p \leq 0.001$ vs G1 (using one-way ANOVA).

Table 1: Effect of the Biofield Energy Treatment on human bone osteosarcoma cell for the assessment of cell proliferation by MTT assay measured at 540 nM.

Assessment of Alkaline Phosphatase (ALP) Activity

Effect of the Biofield Energy Treatment on the level of alkaline phosphatase (ALP) in human bone osteosarcoma cell is presented in Table 2. The absorbance level of ALP in the untreated cells (G1) was 0.212 \pm 0.031. Besides, the level of ALP was significantly increased by 32% in the Biofield Energy Treated group (G3) supplement with 10% charcoal-dextran treated fetal bovine serum (CD-FBS) compared to the G1 group. The positive control (β -estradiol) group (G2) showed a significantly increased the level of ALP by 38%, 35%, and 29% at the concentrations of 1, 10, and 100 nM, respectively compared to the G1 group (Table 3). Overall, the Biofield Energy Treated group (G3) that are supplemented with CD-FBS showed an improved the level of ALP in the human osteosarcoma cells with respect to the untreated group (G1). ALP are membrane-bound ectoenzymes. Human ALP is classified into 4 type's tissue nonspecific, intestinal, placenta, and germ cell. The tissue non-specific type of ALP is

ubiquitously expressed in many tissues, including liver, bone, and kidney, and known as liver bone kidney (LBK) type [31]. The LBK type of ALP is expressed on the cell membrane of hypertrophic chondrocytes, osteoblasts, and odontoblasts. The mechanism in which this enzyme carries out its function to act both to increase the local concentration of inorganic phosphate (Pi), a mineralization promoter, and to decrease the concentration of extracellular pyrophosphate (PPi), an inhibitor of mineral formation [32]. Based on the lots of literatures it has been clear that ALP plays a vital role for bone health, osteogenesis, and calcification process. The enzymes split-up organic phosphate to formed Pi, which thus combined with soluble calcium ion present in the tissue fluids and formed calcium phosphate. After reaching sufficient concentration of calcium phosphate it chemically precipitated into osteoid [33,34]. In this experiment, it was also evident that the Biofield Energy Treated group significantly ($p \leq 0.001$) increased the level of ALP expression.

Group	G1	G2			
		1 nM	10 nM	100 nM	G3
Absorbance	0.212 ± 0.031	0.293	0.287 ± 0.040	0.275 ± 0.045	0.28
		±			±
		0.028			0.016
% Cell Proliferation w.r.t. G1	100	138***	135	129	132***

G1: Untreated cells; G2: Positive control (β -Estradiol) at the concentrations of 1, 10, and 100 nM. G3: Biofield Energy Treated cells. All the groups were included phenol-free DMEM supplemented with 10% CD-FBS. Values are represented as mean \pm SD and the experiment was carried out in triplicates. *** $p \leq 0.001$ vs G1 (using one-way ANOVA).

Table 2: Effect of the Biofield Energy Treatment on human bone osteosarcoma cell for the assessment of alkaline phosphatase enzyme activity measured at 405 nm.

Assessment of Collagen Synthesis

Effect of the Biofield Energy Treatment on human bone osteosarcoma cell and the level of collagen is demonstrated in Table 3. The level of collagen in the untreated cells (G1) was 163.9 ± 2.31 $\mu\text{g/mL}$. Besides, the level of collagen synthesis was significantly increased by 27% in the Biofield Energy Treated group (G3) supplement with 10% charcoal-dextran treated fetal bovine serum (CD-FBS) compared to the G1 group. The positive control (β -estradiol) group (G2) showed a significantly increased in the synthesis of collagen by 19.3%, 10.5%, and 3.0% at the concentrations of 1, 10, and 100 nM, respectively compared to the G1 group (Table 3). Overall, the Biofield Energy Treated group (G3) showed an improve synthesis of collagen in the human osteosarcoma cells with respect to all the groups. The bone health depends on both the quantity as well as quality of bone tissue components. Apart from minerals, the osteoblast and osteoclast cells contain collagen that has an important role in both health. The stability and maturation of collagen in bone occurs in two ways like enzymatic (*i.e.*, activation of lysyl oxidase) and non-enzymatic process through the formation of advanced glycation end products (AGE). In disease state like

osteogenesis imperfecta and osteoporosis there was an abnormality of collagen synthesis, stability and maturation [35,36]. In this experiment, the Biofield Energy Treated group supplemented with CD-FBS was found to enhanced the synthesis of collagen, which might be due to increase formation of AGE, activation of lysyl oxidase or due to post-translational modifications of type I collagen. Another literature described that the osteoblast differentiation and maturation are crucial events in the formation of new bone tissue and for the determination of bone quality due to the synthesis of collagen [37]. Scientists are very keen to find out the ways that can improve collagen synthesis in skeletal disorders patients. Biofield Energy had felt under the canopy of complementary and alternative therapy and already been recommended by National Center for Complementary and Alternative Medicine (NCCAM). About 30% US population are regularly used this therapy for the prevention and treatment of various diseases [38-40]. Besides, here The Trivedi Effect® - Consciousness Energy Healing Treatment modality showed a significant improvement of collagen level in human osteosarcoma cells. Thus, it is assumed that The Trivedi Effect® has the potential to improve the bone health in various skeletal disorders.

Group	G1	G2			
		1 nM	10 nM	100 nM	G3
Collagen ($\mu\text{g/mL}$)	163.9 \pm	195.7	181.2	168.9	208.3
	2.31	±	±	±	±
		9.83	4.75	9.01	7.76
Collagen (%) w.r.t. G1	100	119.3***	110.5***	103	127.0***

Table 3: Effect of the Biofield Energy Treatment on human bone osteosarcoma cell for the assessment of collagen synthesis measured at 540 nm.

G1: Untreated cells; G2: Positive control (β -Estradiol) at the concentrations of 1, 10, and 100 nM. G3: Biofield Energy Treated cells. All the groups were included phenol-free DMEM supplemented with 10% CD-FBS. Values are represented as mean \pm SD and the experiment was carried out in triplicates. *** $p \leq 0.001$ vs G1 (using one-way ANOVA).

Conclusion

The cell viability was assessed using MTT assay in human bone osteosarcoma cells and found that Biofield Energy Healing showed a significantly increased the bone cell proliferation compared to the untreated group (G1). Further, the levels of ALP and collagen synthesis were significantly increased by 32% and 27%, respectively in the Biofield Energy Treated cells (G3) group compared to the G1 group. Overall, the Biofield Energy Treatment significantly enhanced the bone mineralization and differentiation compared to the untreated cells group in human bone osteosarcoma cells. In conclusion, The Trivedi Effect® - Consciousness Energy Healing Treatment might act as an effective bone health enhancer and it can be used as a complementary and alternative treatment for the prevention of various types of skeletal abnormality viz. osteoporosis, limb abnormalities, microcephaly, club foot, polydactyly, congenital hip dislocation, cleft hand and cleft foot, skull abnormalities, anencephaly, syndactyly, encephalocele, etc.

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